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Specific Membrane Receptors: Pathogenetic and Therapeutic Implications in Infectious Diseases

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In the past decade, the level of our understanding of the basic nature of infectious disease processes has changed dramatically. The central idea underlying this change is the realization that there is what might be loosely termed a sociology of infectious diseases, in which host-pathogen interactions of many sorts occur, along with transfer of messages between the interacting cells. Consequently, we must face two of the most fundamental questions being asked in biology today: "What is the nature of specific cellular recognition mechanisms?" and "What is the nature of the language of cell-to-cell communication?" In this presentation I will address myself to these questions as I try to define the role of specific cell-surface receptors in the pathogenesis of infections and the therapeutic implications of this knowledge.

To set the stage, I will discuss the receptor concept in general. This concept has been developed in recent years largely through the work of the endocrinologists studying the peptide hormones. However, although they may have given us the techniques with which to study receptors, we in infectious diseases have given them cholera toxin to play with.

Sir McFarlane Burnet has called the phenomenon of specific recognition between macromolecules a primary characteristic of living matter [1]. This is because recognition phenomena at cell surfaces may be the key to the control of both cellular differentiation and modulation of cell function [2], whether involving neurotransmitters, cyclic nucleotides, hormones, or hormone-like substances such as cholera toxin, as well as immune mediators. These biological regulatory signals have been categorized as Darwinian rather than Lamarckian, that is, they are inductive, not instructive [3]. In other words, communication involves the reception and uncoding of a signal and its translation into an effect that the cell already knows how to accomplish. The communication may fail if there is no receptor available, in which case signals cannot be received, or if the translating or transducer mechanism is missing or defective, in which case the signals may be received but not understood. The basic idea is that of a three-step process: message reception, message translation, and biological response.

The original concepts of cell receptors came from the pharmacology studies of Langley and Clark 50–100 years ago [4]. Drugs were believed to modulate cellular metabolism by acting on "receptive substances" of cells. A classical pharmacologic approach was developed to investigate mechanisms of drug-receptor interactions, employing agonist and antagonist analogues to probe, characterize, and map receptor specificity, and some sort of bioassay to measure the consequences of binding. The approach is still valid today, and it has been successfully used to study many types of cell receptors [4–6].

General Features of Cell Receptors

From the early vague concepts of receptors as unknown structures that mediate the biological effects of different substances on cells, various new and complicated models have been developed. The minimal requirements include a cog-

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nitive element for message reception, an effector element for the output, and a transducer, which links the first element to the second [3]. Whereas the receptor is placed asymmetrically on the outside of the cell membrane, the effector may be at the outer membrane surface, within the membrane, or at its cytoplasmic face. The function of this receptor-effector unit in the modulation of cell processes requires combining-site specificity. The great potential for structural diversity of such sites permits tremendous diversity in receptors, which, in turn, allows specificity of recognition. While each cell type may thus have its own unique complement of different and distinct receptors displayed on the cell surface, it is not necessary to postulate individual and unique transducer-effector mechanisms. In fact, receptor diversity permits conservation with respect to diversity of transducer-effector mechanisms [3]. The detailed chemistry of the receptor site alone can account for species, organism, organ, and cell specificity.

These basic concepts suggest a mathematical treatment of the biochemistry and kinetics of receptor-ligand interactions along the lines of enzyme-substrate models (figure 1). The primary interaction can be depicted as the formation of a ligand-receptor (LR) complex, which occurs at a defined rate, K_1. Like enzyme-substrate reactions, LR complex formation is reversible at a rate determined by K_2. Reversibility permits modulation of biological effects and suggests that binding is not covalent, but rather occurs through weak interactions such as hydrogen bonding, ionic interactions, or van der Vaal's forces [7].

Two conceptual models have been proposed for the subsequent steps in activation and function of the receptor for drugs, hormones, or similar ligands [4]. In the first, which may be called the "squatters" model, occupancy of the receptor serves to activate it, and biological activity is in direct proportion to the number of receptors occupied at any one time. There is turnover of the ligand-occupied receptors, and, ultimately, responsive receptors are regenerated. In the second model, the occupied complex is inert, but the process of binding of ligand to receptor serves to activate the system. The height of the response is thus proportional to the number of complexes formed per unit of time. After this "hit and run" tactic of the ligand, the receptors are recycled. With either model, affinity of the ligand for the receptor is determined by K_1 and K_2, while biological effects are determined by K_3 and K_2. The law of mass action applies to these interactions, and, with use of the squatters model, which is simpler than the second model, equilibrium conditions can be studied to determine association and dissociation constants. The equilibrium constant, equivalent to the total concentration of a ligand at which 50% of the receptors are occupied, can also be calculated.

Experimental Problems

Unfortunately, these constants are only approximations for many reasons, including nonrandom distribution of receptors, the complex surface topology of cells, the proximity of irrelevant steric groups to receptors, and receptor turnover; however, they do indicate the relative affinity of

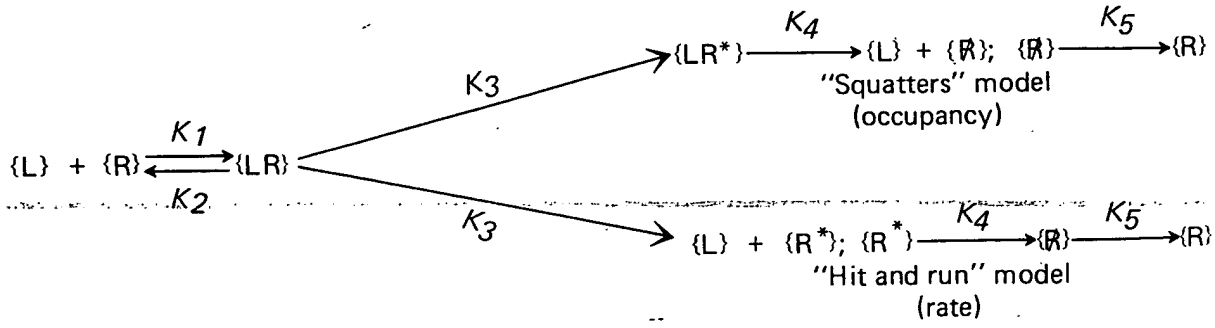

Figure 1. Kinetics of ligand-receptor interactions according to enzyme-substrate models and two mechanisms of activation, occupancy or rate of interaction. L = ligand; R = receptive receptor: R* = activated receptor; R = nonreceptive receptor. Receptor affinity $\sim K_1/K_2$; receptor activity $\sim K_3/K_2$; receptor renewal $\sim K_5$.

the ligand and the specificity of the receptor. Unfortunately, many substances, including bacteria, viruses, and toxins, also stick specifically (that is, with discrimination) to some nonbiological inert surfaces, such as glass or latex. For confirmation that binding of a ligand to a cell surface is both specific and biologically meaningful, several criteria should be satisfied: (*1*) high-affinity binding (permitted at physiologic concentration), (*2*) saturability of binding, (*3*) structural specificity for ligand (agonists and antagonists), (*4*) physiologically relevant tissue distribution, (*5*) reversible binding, (*6*) rapid binding, and (*7*) correlation between binding and bioactivity. In general, specific receptors are present in relatively small numbers, and reception has high affinity but low capacity.

Although binding and bioactivity should be related, this may not be a simple linear relation (figure 2). Three general situations can be defined [4] where this is not the case. In the first situation, a minimal number of receptors must be engaged for effects to be seen. In other words, there will be a threshold for binding before an effect is seen. In the second case, only a fraction of the total receptor population need be engaged in order to see maximal effects. There will be spare receptors present on the cell surface, and occupancy of these equivalent receptors by additional ligand molecules will not elicit any greater response from the cell. It is important to stress the kinetic equivalency of these receptors, for a distinction must be made between high affinity–low capacity receptors and low affinity–high capacity "second sites." The latter may really be physiologically irrelevant, nonspecific sites for adsorption to the cell surface and not for specific reception at all. The role of such sites can be judged best on the basis of whether activation of the effector mechanism follows. In some situations, however, kinetically equivalent receptors may be hidden or masked on the cell surface. These receptors can sometimes be revealed by alteration of the membrane surface by enzymes or detergents. However, if such receptors are not normally available to the ligand, they are not truly "spares." It is possible that they may be involved in other phenomena, such as receptor turnover. The third possible situation in which there is not a simple linear relation between binding and bioactivity would be a combination of a threshold and spare receptors.

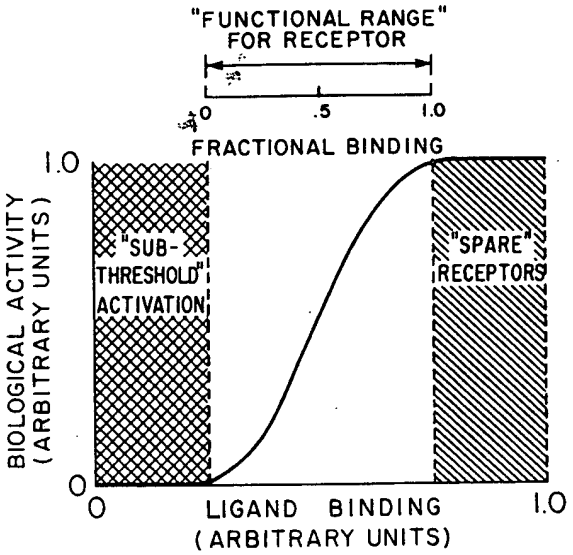

Figure 2. Theoretical relation between ligand binding to its receptor and activation of the biological effector mechanism. A range of receptor binding with no increase in biological activity ("sub-threshold activation") is shown at the left, as well as "spare receptors," to the right, in excess of the number that need be engaged by ligand for full activation of the effector. If biological activity is used to monitor ligand binding, both extreme portions of the curve will be missed, and the functional range measured will be more apparently linear than in reality (adapted from [4]).

Cooperative interactions, both positive and negative, are also possible [4, 8]. A positive cooperative interaction is one in which the initial binding of a ligand in some way increases the binding affinity of the remaining receptors so that more ligand is bound more tightly and more rapidly and with more effect. A negative cooperative interaction is a regulatory feedback loop in which progressive binding reduces the efficiency of binding of additional ligand, thus modulating the biological effects. Since the effect of ligand binding can be either to turn on or to turn off reactions, negative and positive cooperative interactions in binding can each exert augmenting or inhibiting biological effects.

Even with studies of direct binding of a labeled ligand to membrane receptors, there are problems [4]. Results can be altered by any of the following: nonspecific, nonsaturable binding; specific nonreceptor binding (for example, to the test

tube wall); spurious binding at a high concentration of ligand or receptor; nonrandom distribution of receptors on the cell surface or of ligand in the medium; ligand-ligand or receptor-receptor interactions; heterogeneity of receptor or the labeled ligand; cooperative interactions or other allosteric effects; and degradation of ligand or turnover of receptor. It may also be difficult to distinguish between binding of a ligand to receptors and uptake by a selective transport system. This series of problems begins to sound like the adverse reactions section of the package insert of the antibiotics we use—but just as such precautions do not stop us from using chemotherapy in patients, the many confusing factors described above do not preclude laboratory investigation of receptors.

Stereospecific Binding

There are three basic models for stereospecific ligand binding to receptors [3] (figure 3). In the lock-and-key model, a single-step binding process occurs in which all contact points are made simultaneously. Not only does this process require the proper conformation of the ligand for productive binding, but it also imposes an orientational requirement on the system. If the ligand were turned around or flipped over, no binding would occur, and the binding constants could be quite low. In addition, the specificity of binding suggests that there are many contact points and a large surface for the binding interactions. These conditions may be difficult to achieve with a lock-and-key model. If, instead of a single-step process, binding were a sequential interaction of the receptor and ligand, as in the second and third models shown in figure 3, there would be far less stringent orientational and conformational requirements for the process. Induced fit of either receptor or ligand would need only an initial contact at one point or small region, which, although a weak interaction, could lead to a series of conformational changes of one or both components and, as a result, a firm and productive binding of the entire ligand to the entire receptor. Since the rate-limiting step in the induced-fit process would be the initial contact, there would not be much difference in the association constant or the equilibrium constant between the induced-fit process and the lock-and-key model, because the final energy state achieved by either path would be the same.

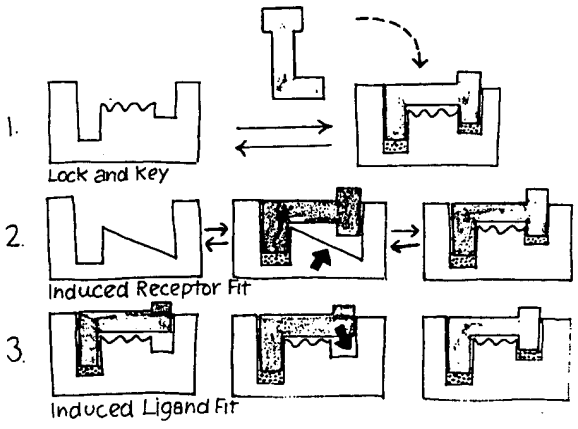

Figure 3. Models for stereospecific ligand binding to receptors. (*1*) Lock and key: single-step binding process with all contact points made simultaneously. (2) Induced receptor fit: initial binding at one point followed by alteration of receptor (arrow) to fit ligand (*3*) Induced ligand fit: initial binding at one point followed by alteration of ligand (arrow) to fit receptor. Note that the final energy state achieved with all three models is identical (adapted from [3]).

Nature and Arrangement of Recognition Units

The plasma membrane is now well known to be a fluid, mobile, lipid bilayer [9]. However, we rarely consider the fact that it is functionally and structurally asymmetric. The external face carries the molecular markers of cellular uniqueness as well as the recognition sites for extracellular regulatory molecules or for cell-to-cell contacts, i.e., the receptors we are talking about. Proteins and sugar-containing glycoproteins and glycolipids are also part of the membrane [10]. Some are intrinsic or integral components that are largely within the lipid bilayer itself. These must be hydrophobic molecules, and they can be released only by disrupting the hydrophobic interactions within the membrane, for example, by detergents. Some may span the membrane from face to face; however, the hydrophilic carbohydrate groups that must stick out do so only into the external aqueous phase, for no carbohydrate is found on the cytoplasmic face of the membrane [10]. The glycolipids are similarly oriented, and, therefore, all of these components of the

membrane have both buried hydrophobic and exposed hydrophilic groupings. Such membrane components are termed amphipathic.

The presence of the sugar sequences of these amphipathic glycoproteins and glycolipids at the external cell surface places them in a key position to function in reception phenomena. In fact, polysaccharide units are good candidates for participation in such recognition functions of receptors. In addition to their strategic placement, great structural diversity is possible. A simple glucose disaccharide can exist in 11 distinct forms, and a glucose trisaccharide yields 176 anomeric configurations [10]. A mixed trisaccharide would, obviously, yield many more possible structural variants. Typically, oligosaccharides of mammalian cell membranes are composed of between four and 20 monosaccharide residues, most commonly of glucose, galactose, mannose, fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and *N*-acetylneuraminic acid. The specific sequence and arrangement of these sugars, then, could provide the basis for configurational complementarity between the recognizer and the recognized, and, in a stereospecific fashion, allow a noncovalent, high-affinity, reversible interaction with sensitivity, selectivity, and speed.

Receptors in Infectious Diseases

In a generic sense we have known about specific receptors for a long time in infectious diseases. For over two decades virologists have studied the concept that receptors for viruses on the surface of target cells may mediate attachment (the initial stage in invasion) [11]. Somehow, the rest of the infectious diseases field did not get the message clearly until very recently.

To illustrate the mechanisms involved, I therefore wish to consider the attachment of two other distinct organisms to their target tissues. In one, a rapid and obvious effector response to attachment occurs, whereas the second is seemingly without effector output.

Receptors for Malaria Merozoites

Louis Miller, who addressed the Infectious Diseases Society of America two years ago on the prospects and problems in malaria vaccine development [12], and his colleagues at the National Institutes of Health (Bethesda, Md.) have been studying the process of invasion of erythrocytes by malaria merozoites for the past few years. Their work has substantiated the suggestion made 25 years ago by McGhee [13] that erythrocyte invasion is initiated by a specific interaction of the plasmodium with the surface of the red blood cell. Working first with *Plasmodium knowlesi,* an agent of rhesus monkey malaria that can also infect humans, Dvorak et al. showed that the process of invasion involves adherence of the apical end of the organism to the erythrocyte, followed sequentially by deformation of the erythrocyte surface, a rebound to the normal shape, invagination of the merozoite into a developing vesicle derived from the red blood cell membrane, and finally the resealing of the erythrocyte surface [14]. Adherence is associated with a thickening of the contacting membranes, visible by electron microscopy, occurring only with erythrocytes from species sensitive to the plasmodium [15]. Suspecting that the relevant surface feature of the red blood cell could be a blood group substance, and knowing that red blood cells from certain black people were resistant to *P. knowlesi* in vitro, Miller et al. correlated susceptibility of red blood cells to invasion by this species with the presence or absence of certain blood group substances (table 1). They found that resistance in humans was associated with absence of Duffy determinants, a common finding in blacks, but a rare situation in other populations unless genetic admixture had occurred [16]. Red blood cells from three

Table 1. Susceptibility of erythrocytes to merozoites of *Plasmodium knowlesi* and *Plasmodium falciparum.*

Source or treatment of erythrocytes	Susceptibility to	
	P. knowlesi	*P. falciparum*
Caucasian, Duffy-positive	Yes	Yes
Black		
Duffy-positive	Yes	Yes
Duffy-negative	No	Yes
Nonblack, Duffy-negative	No	Not Done
Duffy-positive, treated with		
Chymotrypsin	Reduced	Yes
Trypsin	Yes	Reduced
Neuraminidase	Yes	Reduced
Rhesus monkey	Yes	No

NOTE. Data are from [16-18].

nonblack, Duffy-negative individuals (two American Indians and one white Australian) were also studied, and these cells were appropriately refractory to *P. knowlesi* [17]. Treatment of sensitive, Duffy-positive erythrocytes with chymotrypsin, which removes the Duffy determinants, reduced susceptibility to *P. knowlesi,* and treatment of these cells with trypsin or neuraminidase, which do not remove the Duffy determinants, did not affect susceptibility [18]. When *Plasmodium falciparum* was studied, however, the results were quite different and indicated the specificity of the merozoite-erythrocyte interaction. In contrast to the findings with *P. knowlesi,* both Duffy-negative and Duffy-positive cells, even after treatment with chymotrypsin, were susceptible to *P. falciparum,* whereas treatment of Duffy-positive cells with trypsin or neuraminidase reduced the susceptibility [18]. In other words, in the same red blood cell, different surface features must mediate the invasion of the two strains of malaria parasite.

Miller and colleagues [19] have recently shown that addition of cytochalasin B to Duffy-positive erythrocytes and *P. knowlesi* permits attachment of the merozoites to the erythrocytes, but not invasion (table 2). Much to their surprise, Miller et al. found that when cytochalasin B was added to merozoites and Duffy-negative erythrocytes, the attachment of merozoites to the red blood cells was equivalent to that observed with Duffy-positive cells. In other words, the Duffy antigens themselves are probably not involved in merozoite adherence, although something associated with the Duffy blood group substance is in some way involved in invasion. During invasion, a circumferential zone of attachment, with a localized thickening of the red blood cell membrane, develops at the junction of the invagination site of the red blood cell membrane and the merozoite [15]. As the parasite is progressively enveloped within a vesicle derived from the red blood cell surface, this junction moves along the confronting membranes in a way so as to maintain its position in the plane of the surrounding erythrocyte surface. Since the red blood cell is not phagocytic and the merozoite is not motile, this moving junction may well be a key feature in the invasive process and not just a morphological detail. Both attachment and junction formation are seen in Duffy-positive cells in the presence of cytochalasin B, but only attachment is observed with Duffy-negative cells. However, trypsinization of these Duffy-negative erythrocytes permits both attachment and junction formation in the presence of cytochalasin B. This finding is consistent with the observed merozoite invasion of the trypsinized Duffy-negative erythrocyte in the absence of cytochalasin B.

Miller et al. concluded that the red blood cell has a *P. knowlesi* reception site, of unknown composition, which is not Duffy-associated [19]. Attachment is followed by a complex process including formation of a moving junction and invagination of the membrane. These events, in some way, usually involve Duffy-associated or linked membrane components. It is reasonable to propose that in this system, the specific attachment occurring between merozoite and red blood cell in some fashion triggers the membrane events described. Treatment of the merozoite with cytochalasin B blocks junction movement; hence, if there is a definable effector involved in the invagination step, it is more likely to be found in the merozoite membrane than in the red blood cell. One might speculate further that the transducer is located in the erythrocyte membrane, available to interact with the parasite membrane when Duffy antigens are present or, in Duffy-negative cells, when something blocking the transducer is removed by trypsin. It is quite clear, however, that receptor-ligand interactions specify attachment and participation in the subsequent events in invasion of erythrocytes, and thus are keys to the pathogenesis of malaria.

Table 2. Interaction of *Plasmodium knowlesi* merozoites and erythrocytes.

Erythrocytes	In presence of cytochalasin B		Invasion (in absence of cytochalasin B)
	Attachment	Junction formation	
Rhesus monkey	Yes	Yes	Yes
Duffy-positive	Yes	Yes	Yes
Duffy-negative	Yes	No	No
Duffy-negative treated with			
Trypsin	Yes	Yes	Yes
Chymotrypsin	No	No	No
Guinea pig	No	No	No

NOTE. Data are from [19].

Receptors and Ligands of Bacterial Adherence

Adherence of enterotoxigenic *Escherichia coli* to the proximal small bowel via specific receptors is a recently described trait necessary for virulence of these organisms. As far as I know, this adherence is not associated with any direct effector response, i.e., it appears to be simply ecologic in nature, providing the organisms a way to establish colonial growth in the flowing stream of the intestinal contents. Colonization is, of course, a necessary condition for toxin production, the prime mover of water and electrolytes in the pathogenesis of diarrhea.

The most thoroughly studied system in which the adherence mechanism of *E. coli* is at least partially understood is the pathogenesis of diarrhea caused by a porcine strain of *E. coli* that bears a protein capsular surface antigen called K88. The classical studies of Williams-Smith and colleagues in England first established the role of K88 in the pathogenesis of diarrhea of neonatal piglets [20]. It was observed that large numbers of *E. coli* were present in and adherent to the proximal small bowel of pigs dying of diarrhea caused by toxigenic *E. coli,* but not when avirulent strains were employed. Most of the virulent strains were observed to possess K88 antigen, previously described as a hemagglutinin [21]. Because production of both toxin and K88 antigen is regulated by plasmid DNA, the role of toxin and K88 as virulence factors either singly or in combination was evaluated. Beginning with a K88-negative and toxin-negative porcine strain of *E. coli* (serotype O8:K40), Linggood and Williams-Smith [22] first added the toxin plasmid to the organism (table 3). Although in vitro this strain produced toxin capable of causing secretion of intestinal fluid, it was avirulent when fed to the intact animal, and it failed to colonize. Insertion of K88 alone in the organism led to a marked colonization of the proximal intestine with minimal alteration in the bowel habits of the animals, loosely described by the authors as diarrhea, but mild and nonfatal. Simultaneous presence of both factors produced a colonizing strain of *E. coli* that caused severe diarrhea with high mortality in most animals tested.

Table 3. Effect of addition of plasmids of K88 antigen and toxin on the virulence of a strain of *Escherichia coli* enteropathogenic for pigs.

Plasmid		In vivo observations	
Toxin	K88	Intestinal colonization	Clinical disease
No	No	No	No
Yes	No	No	No
No	Yes	Yes	Mild
Yes	Yes	Yes	Severe

NOTE. Data are from [22].

Gibbons, Sellwood, and coworkers, also in England, added a dimension of credibility to the K88 story when they showed that K88-positive strains, but not K88-negative strains, adhere in vitro to brush borders prepared from the small intestine of pigs [23]. Moreover, K88-positive strains grown at 18 C, which is apparently room temperature in England, fail to produce the K88 antigen and do not adhere.

If K88 antigen is the ligand on the bacterial surface, it should bind with a receptor on the intestinal cell surface. Gibbons, Sellwood, and colleagues have recently provided us with good evidence of this receptor [24]. Employing adherence of K88-positive *E. coli* to brush borders as an in vitro assay for the K88 receptor, they showed that certain animals possess receptors, whereas others do not, or do not have them available on the surface of the intestine. This variation was considered to represent two phenotypes in the pig population studied: one, designated susceptible, with available receptors on the intestinal brush border, and the other, designated resistant, without detectable receptors. When these investigators examined a number of litters sired by different boars, they found that some boars produced only susceptible offspring, some produced only resistant offspring, and some produced mixtures of susceptible and resistant offspring. These workers also examined K88 receptors in litters produced from matings in which one or both of the parents were subsequently killed to allow phenotyping (table 4). When one or both parents were susceptible, litters contained either all susceptible or a mixture of susceptible and resistant offspring. Matings in which both parents were known to be resistant produced only resistant offspring. The simplest genetic interpretation of these data is a two-allele system, consisting of the susceptible (dominant) and resistant (recessive) traits,

Table 4. Distribution of K88 antigen receptor phenotypes in litters from pigs with known phenotypes.

Parental phenotype, no. of litters	Phenotype	
	Susceptible (S/?)	Resistant (ss)
S X s		
8	36*	32
2	23	0
s X s		
9	0	84
S X ?		
4	46	0
1	6	2
s X ?		
5	50	0
16	71	71
5	0	44

NOTE. Data are from [24]. The phenotypes were designated susceptible (S) (with detectable receptors for *Escherichia coli* K88 antigen on the intestinal brush border) and resistant (s) (without detectable receptors).

*Data are given as number of piglets.

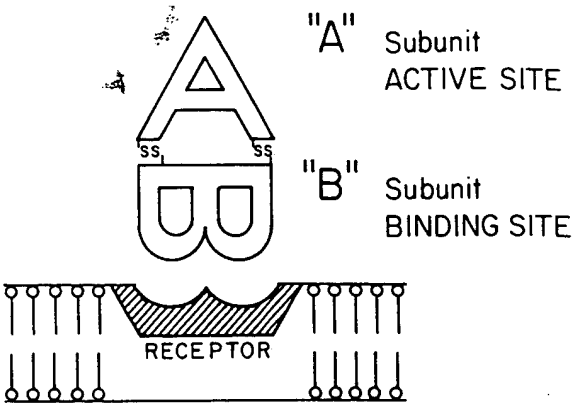

Figure 4. General A-B subunit model of toxin structure and interaction with cell membrane receptors. The "B" polypeptide is complementary to receptor and determines binding specificity and affinity; "A" polypeptide, linked to B subunit by disulfide bridges, is the catalytic or active portion of the molecule involved in activation of the appropriate effector mechanism. Toxins that exemplify this type of interaction are those from *Bordetella pertussis, Pseudomonas aeruginosa, Vibrio cholerae*, and the plant toxin, ricin.

in which the laws of Mendelian inheritance operate. The nature of the K88 receptor is still unknown, but its existence in the brush border membrane of the susceptible pig has been clearly demonstrated.

Toxin Receptors and Effector Mechanisms

Cholera toxin is one of a number of biologically important substances, including microbial and plant toxins, which are built as two-component molecules, the A-B subunit model represented in figure 4. In these toxins the B subunit is a separate peptide chain that carries out the receptor-recognizing function, fitting in complementary fashion into the receptor on the surface of the sensitive cell. The B subunit is linked by disulfide bonds to a separate peptide chain, the A subunit, which is the physiologically active portion of the molecule. Isolated A or B subunits exert no toxic effects on intact cells—they must be linked together as the holotoxin to be biochemically active.

Study of the binding of cholera toxin was immeasurably advanced by preparation of highly purified, biologically active toxin and isolated subunits and labeling of the toxin with ^{125}I [25]. With these reagents it could be shown that labeled cholera toxin at low concentrations ($\leq 10^{-10}$ M) is quantitatively bound, the process being completed within 15 min at 4 C or 5 min at room temperature (~24 C). The equilibrium constant derived from these studies, about 5×10^{-10} M, is equivalent to those for polypeptide hormones binding to their receptors. Isolated B subunit and unlabeled holotoxin are equally effective and potent competitive inhibitors of binding of ^{125}I-labeled toxin. In many studies with membranes from many cell types, it has been demonstrated that isolated B subunit binds to the bona fide toxin receptor with kinetics and affinity identical to those of native toxin, but does not cause any alteration in physiology. Simple squatting of the binding moiety on the receptor is insufficient to activate the effector, which, in the case of cholera toxin, is the adenylate cyclase enzyme system.

In 1971 the key to the nature of the toxin receptor was provided by van Heyningen et al. [26], who demonstrated that mixed gangliosides from bovine brain competitively inhibited the biological activity of cholera toxin. There is now a wealth of data available showing that the monosialosyl glycolipid G_{M1} ganglioside (galactosyl-N-acetylgalactosaminyl [sialosyl] lactosylceramide) is the most avid toxin binder of all the gangliosides and that cell sensitivity is related to the

presence and amount of G_{M1} in the membrane. This relation between cell sensitivity and G_{M1} content may be illustrated by studying a series of glycosphingolipids of increasing complexity and number of substituents and comparing them with respect to effectiveness as competitive inhibitors of binding of [^{125}I]cholera toxin, as Cuatrecasas has done [27]. Compounds that are simpler than G_{M3} (monosialosyl lactosylceramide) are not at all effective as inhibitors; however, when *N*-acetylneuraminic acid (sialic acid) is joined by 3,2 linkage to the galactose moiety of the lactose, and then *N*-acetylgalactosamine and galactose are added in sequence, inhibitory potency increases about 25-fold in G_{M2} (monosialosyl-*N*-triglycosylceramide) and 2,500-fold in G_{M1}. Further modification of G_{M1} (for example, by addition of a second sialic acid on the terminal galactose in GD_{1a} [disialosyl-*N*-tetraglycosylceramide] or a third sialic acid in G_{T1} [trisialosyl-*N*-tetraglycosylceramide]) reduces the activity by 25- to 500-fold. These data also show the importance of the carbohydrate units for specificity and avidity of binding. The glycolipid cholera receptor is pronase- and trypsin-resistant, but it is removed by extraction with chloroform-methanol.

Gangliosides are common amphipathic membrane constituents with a hydrophobic domain that is inserted into the membrane, orienting the hydrophilic carbohydrate units out on the membrane surface [10]. The widespread distribution of G_{M1} in various cell types accounts for the simultaneous exquisite discrimination and promiscuity of cholera toxin—wherever there is G_{M1} ganglioside, there cholera toxin will bind. Indeed, labeled cholera toxin has been used as a probe to detect and quantitate G_{M1} ganglioside in membranes of cultured and transformed cell lines, and it is 10^3–10^4 times more sensitive in this detection than chemical methods [25]. Toxin binds stoichiometrically to G_{M1} ganglioside in a 1:1 molar ratio. Incubation of certain cells in medium containing G_{M1} ganglioside results in the incorporation of the exogenous G_{M1} molecules into the membrane. It can be shown that there is a nearly stoichiometric increase in toxin-binding capacity of those cell membranes, but no alteration in the affinity of binding. In certain instances sensitivity to biological effects of cholera toxin increases simultaneously as exogenous G_{M1} is taken up into the membrane; this increase in sensitivity occurs when the number of usual binding sites is limited and rate-limiting for cyclase activation [28], but is the exception with cholera. In most instances the number of G_{M1} receptors exceeds by more than 50-fold the number required to be in binding interaction with the toxin to produce half-maximal activation of cyclase. Moreover, all of these receptors are equivalent: structurally, kinetically, and functionally they are true "spares," and only a small number need to be occupied with toxin to produce biological effects.

Bennett et al. [29] have advanced the mobile receptor hypothesis for this activation process, a two-step mechanism in which the regulatory molecule binds to its receptor and then the complex migrates to the site of the effector enzyme in order to activate it. In the absence of the ligand, the receptor and effector are not directly associated. Indeed, this is the usual state in the target tissue for cholera toxin, the intestinal cell, a morphologically polarized cell in which receptors are in the brush border membrane at the bowel lumen and adenylate cyclase is in the lateral cell membrane. Binding of toxin, followed by aggregation of receptors, was observed in lymphocytes when fluorescent toxin was used [30]. This patching phenomenon requires the toxin to be multivalent, and it is prevented by antitoxin, which simultaneously prevents activation of cyclase. In lymphocytes, patching of cholera toxin is followed by capping, which demonstrates that G_{M1} receptors in these cells can diffuse laterally in the plane of the membrane. Thus, it is not unreasonable to propose that in the intestinal cell, where the receptors are concentrated in a region of the cell devoid of effectors, aggregation and migration of receptors are involved in delivery of toxin to cyclase. The lag phase encountered after the application of cholera toxin to intact cells could be the time needed for aggregation of receptor-toxin complexes, lateral diffusion of these complexes within the confines of the cell membrane, and interaction of the A subunit with adenylate cyclase.

In contrast, the binding of shigella toxin to cell membranes differs in a number of respects from that of cholera toxin. For some time now

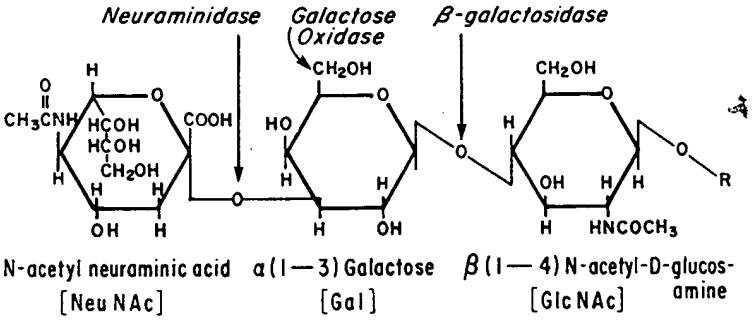

Figure 5. Common terminal oligosaccharide unit in glycoproteins and sites of action of neuraminidase, galactose oxidase, and β-galactosidase.

my laboratory has been investigating a cytotoxin produced by all species of *Shigella* in a model system employing HeLa cells in culture. This cytotoxin is associated with the activity of an enterotoxin (intestinal secretion) and neurotoxin (death). We have learned enough over the past few years in this model system that if a HeLa cell should come to my office complaining of dysentery, I can offer a rather effective therapeutic regimen.

First, there is a membrane receptor present on cells sensitive to shigella toxin, but not on resistant cells [6]. The receptor is pronase- and trypsin-sensitive, and it is susceptible to destruction by lysozyme. These data suggest that the shigella toxin receptor is a glycoprotein, the carbohydrate portion of which is a lysozyme substrate. Because of the enzymatic specificity of lysozyme, there are not too many possibilities for this postulated carbohydrate. We have tested many monosaccharides and oligosaccharides as hapten inhibitors of shigella toxin; however, only the lysozyme substrates of β-1 → 4-linked *N*-acetylglucosamine, chitotriose, and chitotetraose are effective—all other sugars, as well as glycoproteins and gangliosides, that we have studied are inactive. These findings are supported by experiments using plant lectins as probes, including phytohemagglutinin (PHA) and concanavalin A (con-A), which do not bind to the postulated receptor oligosaccharide. Neither PHA nor con-A is an inhibitor of toxin, whereas wheat germ agglutinin, known to bind preferentially to the oligosaccharide postulated as receptor, effectively blocks toxin uptake.

In addition, there is a population of "masked" toxin receptors on the surface membrane of the HeLa cell.[1] There is a common sequence of sugars in the terminal portion of the oligosaccharide side chain of many glycoproteins (figure 5). Several enzymes are capable of acting on these sugars, for example, neuraminidase and β-galactosidase, which hydrolyze specific bonds, and galactose oxidase, which converts the C-6 of galactose from an alcohol to an aldehyde. Treatment of HeLa cells with either neuraminidase or galactose oxidase does not affect shigella toxin activity —β-galactosidase does. We can detect three effects after exposure of HeLa cells to β-galactosidase: (*1*) enhancement of toxin activity, (*2*) potentiation of the action of lysozyme on the receptor, and (*3*) augmentation of the receptor blockade afforded wheat germ agglutinin. The cells gradually return to base-line responsiveness to toxin after treatment with β-galactosidase, but this takes several hours. However, if we add 10 mM galactose to the medium, the restoration occurs within minutes. This effect is substrate-specific—other monosaccharides do not substitute for galactose. Therefore, it is highly likely that we are supplying a specific substrate to a galactosyltransferase on the cell exterior that can rapidly restore the involved surface glycoproteins to the galactose-terminal normal state for HeLa cells following the exposure of cells to β-galactosidase.

With this evidence for galactose-terminal masked receptors for shigella toxin in HeLa cells, we have begun to reinvestigate cells previously shown to be resistant. The Y-1 adrenal cell, for example, becomes sensitive to shigella toxin when pretreated with β-galactosidase (G. T. Keusch and M. Jacewicz, unpublished observations). The capacity of shigella strains to produce this enzyme may have pathogenetic signifi-

[1] G. T. Keusch and M. Jacewicz, "Pathogenesis of Shigella Diarrhea. IX. Unmasking of Shigella Toxin Receptors on the HeLa Cell Membrane by β-Galactosidase," manuscript in preparation.

cance, functioning to expose toxin receptors on the surface of the intestinal cell as it does on HeLa or Y-1 cells. We believe that the effector sequence in the HeLa cell cytotoxicity system involves an active endocytic transport process that brings the toxin into the cytoplasm where it inhibits ribosomal function [31]. Cytotoxicity, then, is the ultimate expression of an inhibition of protein synthesis analogous to that seen with ricin, a toxic plant lectin [32].

With respect to their receptors, we can contrast cholera and shigella toxins (table 5). They are distinct in their chemical nature, oligosaccharide structure, enzyme sensitivity, lectin binding, and effector linkage. In both cases, however, the pathophysiological event follows the specific binding of toxin to receptor.

Therapeutic Implications

To discuss the therapeutic implications of these specific receptors as binding sites for ligands that are key virulence determinants of infection, I will use bacterial enteritis as a prototype for the infectious process, and the examples of receptor-ligand interactions already cited as the data base. The first principle might be called receptor megatherapy. If the stereospecific structure of the bacterial or toxin receptor is known, it is possible that large quantities of it could be introduced by mouth in some particulate form, for example, coated onto a nonabsorbable bead such as cellulose or latex. In the case of cholera toxin and its B subunit, complementary receptive structures are present on the cell surface, the specificity for which resides in G_{M1} ganglioside. If the artificial receptor were to have enormous numbers of open available binding sites, it could act as a competitive inhibitor of toxin when present in the intestinal lumen. Every toxin molecule that would bind to the receptor-coated bead would be taken up in a nonproductive binding interaction. Chemical modifications might, in fact, produce an acceptor molecule with an even higher affinity for ligand than that of the natural receptor, compete for toxin molecules already bound to the cell surface, and perhaps remove the biologically active subunit from the membrane after initial binding of holotoxin to the membrane but before catalytic stimulation of the effector.

Table 5. Properties of receptors for toxins of *Vibrio cholerae* and *Shigella*.

Property of receptor	Toxin	
	V. cholerae	*Shigella*
Chemical nature	Glycolipid	Glycoprotein (?)
Hapten inhibitor	G_{M1} ganglioside	Chitotriose
Lectin inhibitor	?	Wheat germ agglutinin
Effect of		
Trypsin, pronase	None	Destroys
Lysozyme	None	Destroys
β-Galactosidase	None	Unmasks

The selectivity of receptor-ligand binding suggests a second therapeutic modality, that of receptor blockade, in which a specific binding unit with no capacity to activate the effector mechanism is employed to occupy the available surface receptors. A clear example would be blocking of the binding of cholera toxin by occupation of the available receptor sites with the isolated binding subunit B of the toxin. Because there are many more receptors on cells than need to be occupied with toxin for full activation of adenylate cyclase, the excess receptors being fully functional spares, it will undoubtedly be necessary to use a high concentration of the B subunit to obtain a clinically effective blockade. This requirement is quantitatively magnified by the turnover of receptor and ligand, which provides a time frame of reference for continuous presence of blocker. However, there is no theoretical limitation to production of this biological product; recombinant DNA techniques could be used to produce a biofactory. Indeed, one could conceptualize the insertion of DNA for B subunit into a proximal intestine–colonizing, nontoxigenic strain of *E. coli*, which might even bring the biofactory from the boondocks to the bowel itself, thus satisfying the need for continuous administration.

The property of reversibility of receptor-ligand interactions introduces a third approach, elution therapy. The model here is that of affinity chromatography in which substances may be purified by filling a column with an insoluble matrix capable of specifically binding the desired material. The nonbinding contaminants can then be washed off the column with buffer. By addition of a high concentration of the specific sugar involved in the formation of the weak interactions

of binding, the bound molecule can be dissociated from the matrix, and the purified compound can be collected in a test tube. The gut is a good candidate organ for elution therapy—it at least has the appropriate general shape of a column. Indeed, in experimental systems, Jones and Freter [33] have shown that L-fucose can dissociate most *Vibrio cholerae* cells adherent to isolated intestinal brush borders; this observation suggests a role for this sugar in the cell-to-cell binding interaction.

A fourth possibility is suggested by the studies mentioned above in which the activity of shigella toxin receptors of HeLa cells can be manipulated by removing or replacing a terminal galactose on the cell surface. In a similar fashion it may be possible to alter an intestinal receptor in vivo by supplying an appropriate enzyme to modify or remove the key terminal or internal sugars. Alternatively, a substrate could be locally supplied to accomplish the same goal through covalent linkage to the surface oligosaccharide, using endogenous glycosyltransferases already present and available in the outer surface of the cell membrane. This principle might be called receptor modification, and it has already been demonstrated in vitro for the hepatic binding receptor for asialoglycoproteins circulating in the serum of mammals [34]. In this system, treatment of the receptor with neuraminidase removes receptor function, but this can be restored by supplying uridine diphosphate-*N*-acetylneuraminic acid as substrate for an endogenous sialyl transferase [35]. In other words, restoration of a sialic acid terminal glycoprotein is associated with recovery of its receptor activity.

Finally, I mention in passing the possibility of a fifth approach, that of mounting an immunological attack against the surface features of the pathogen that are integral to the binding interaction, whether the organism bears the receptor that recognizes host oligosaccharides or host membranes bear the receptors that recognize the organism. Since the mammalian receptors are probably used for purposes other than helping microbes cause infectious diseases, it might be quite dangerous to try the converse approach—there already is the example of autoimmune disease due to antireceptors in certain insulin-resistant diabetics [36].

Epilogue

In returning to the real world, therefore, it is imperative to realize that great caution is required in implementing these approaches; however, there are also obvious and exciting great expectations for these therapeutic modalities. In a few years it may well be possible for someone listening to this presentation to describe the conquest of one of the ancient infectious plagues of humanity by one or another of the approaches I have outlined—without the use of an antibiotic, antiviral, antihelminthic, or antiprotozoal agent. That one of you should accomplish this is as it should be. We are part of an unfolding process of inquiry in which our task as scientists is quite clear. It has been well stated in another context by a subject of Robert Coles recorded in his book *Children of Crisis* [37]: "I wonder about the answers. I'll ask myself one day, and I'll ask myself the next day, and I'll just decide not to be too sure, just keep on asking."

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